FINAL REPORT

19960413

BIOTECHNOLOGY FOR SASKATCHEWAN PULSE CROP IMPROVEMENT

December 2002
Funded by the Agri-Food Innovation Fund

Prepared by: Plant Biotechnology Institute

AGRI-FOOD INNOVATION FUND

Canada

Saskatchewan

National Research Council Canada
Plant Biotechnology Institute

Conseil national de recherches Canada
Institut de biotechnologie des plantes

NRC/PBI FINAL PROGRESS REPORT ON AFIF PROJECT #96000413 "BIOTECHNOLOGY FOR SASKATCHEWAN PULSE CROP IMPROVEMENT"

September, 2002

Compiled by Drs. Pierre Fobert and Patricia L. Polowick
National Research Council of Canada
Plant Biotechnology Institute
110 Gymnasium Pl. Saskatoon SK S7N 0W9

Dr. Pierre Fobert _____

Dr. Patricia L. Polowick P L. Polowick

(a) Summary

The objective was to apply biotechnology in the development of strategic technologies and enhanced germplasm for the improvement of Saskatchewan pulse crops. To this end we (1) developed transgenic pea germplasm with economic levels of herbicide tolerance; (2) identified new genes capable of turning on disease resistance, produced and tested transgenic peas containing some of these genes; (3) determined that foreign genes are stable and functional in transgenic peas, have little or no effect on crop performance and outcross at a very low frequency (%); (4) demonstrated that a 'jumping' gene from maize is active in peas and could be used for mutagenesis and isolation of economically relevant genes.

(b) Technical Report

Sub-Project 3: Transgenic Peas with Liberty Tolerance and Anti-Fungal Proteins
Drs. J. D. Mahon, A. Vandenberg and P. L. Polowick

Background and Objectives

Decreased yield and seed quality caused by fungal pathogens, poor competition against weed infestation, and the high cost of chemical weed control are major problems in Western Canadian pulse crop production. In some crops, the addition of herbicide tolerance genes and anti-fungal proteins, through biotechnology, has ameliorated some of these problems. The ability to genetically transform peas opens these technologies to pulse crops. The specific objectives of this sub-project were to introduce genes for Liberty®-tolerance and anti-fungal proteins into pea germplasm, to assess the transgenic plants for insertions, genetic stability and expression of the foreign genes, and to develop and test for economic levels of herbicide tolerance and disease resistance in the field.

Project activities related to milestones, achievements

Note: European concerns about transgenic plants checked any plans for the commercialization of transgenic, herbicide-tolerant peas at the present time. Therefore, despite success in developing transgenic peas containing the AgrEvo phosphinothricin acetyl transferase (*pat*) gene (pHOE6AC) and demonstrating agronomically effective levels of tolerance to the herbicide Liberty® (active ingredient, L-phosphinothricin; L-PPT), this sub-project was terminated in March 1999. By this time, many of the milestones had already been achieved.

Milestone #1: T_0 plants of 'Greenfeast' (GF) with *pat* recovered and tested (Year 1).

Achievement: Initially, the *Agrobacterium* method of transformation modified from Schroeder *et al.*, 1993 (Plant Physiol. 101-751) was used to introduce the *pat* gene to explants from Greenfeast, the cultivar on which much of the methodology was established. Over the course of the project, 173 T_0 GF plants were recovered from 23 independent transgenic events. The transgenic plants were assessed by PCR, Southern hybridization analysis and an ELISA expression assay for the PAT protein. These methods confirmed the presence, integration and expression of the gene in the recovered plants.

Milestone #2: Initial T_0 transformants of *pat* in CDC breeding lines recovered (Year 1).

Achievement: Aware that 'Greenfeast' did not have agronomic qualities that would be of use to Western Canadian pea producers, we proposed to introduce the *pat* gene into germplasm adapted to local growing conditions. Four advanced breeding lines from CDC (including CDC 'Mozart') were co-cultivated with *Agrobacterium* strain LBG85 containing the AgrEvo *pat* gene. In Year 1, a total of 78 transgenic plants from 37 independent transformation events were recovered. The presence of the gene was confirmed by PCR and Southern hybridization and PAT protein content of tissues ranged from 0 to 3000 pg PAT/μg total protein. The best plants in each genotype were chosen to produce lines homozygous for the *pat* gene.

Milestone #3: GF-*pat* plants crossed with CDC breeding lines to produce F1 plants (Year 1).

Achievement: Some of the earlier generation seed was provided to the CDC for parallel crossing of each line into adapted CDC germplasm. Transformed 'Greenfeast' lines PLP18 and DB131 were each crossed to 9 advanced breeding lines from the CDC pea improvement program. A backcrossing program was initiated in the phytotron in the fall of 1997. Five lines were chosen as backcross parents. F_1 plants from each T-Greenfeast x CDC cross were used as pollen donors. BC_1 F_1 seeds were planted in the phytotron in the winter of 1998 to use as pollen donors for the second backcross series. A summer crossing block was planted at the Kernen Crop Research Farm in 1998 to develop an F_2 population for later seed increase.

Milestone #4: Initial T3 plants of GF-*pat* recovered and tested (Year 2).

Achievement: Two independent transgenic plants (PLP18 and DB131) were selected on the basis of their levels of pat gene expression, as determined by the PAT protein assay (1143 and 1301 pg PAT/μg total protein, respectively). T_1 progeny were examined for the presence of the pat gene and Chi square analyses showed no significant deviation of the observed ratios from the 3:1 Mendelian segregation expected for a single insertion event. T_2 plants were examined using leaf painting with the herbicide to identify 6 putative homozygous T_1 PLP18 plants and 4 homozygous T_1 DB131 plants. PCR and leaf painting assays were used on a total of 126 T_3 plants, and verified the homozygosity of 5 lines of PLP18 and 3 lines of DB131. Seed from each independent genotype was bulked and advanced to produce T_5 transgenic seed used for a confined Liberty® spraying trial during the summer of 1998 (see Milestone #8).

Milestone #5: Transformation of CDC-*pat* completed (Year 2).

Achievement: Compared to the initial report in Milestone #2, the *pat* gene was introduced into additional CDC germplasm. Overall, total of 285 transgenic plants from 63 independent transformation events were recovered in six CDC breeding lines.

Milestone #6: Second generation of CDC-*pat* breeding material produced and assessed for *pat* (Year 2).

Achievement: Selected T_1 seed was planted and plants with the *pat* gene were identified by leaf painting with Liberty®. T_2 progeny were planted to select homozygous lines. Generally, plants with high expression of the *pat* gene were chosen for further study, especially in line 9705 ('Mozart') which was most advanced in the varietal registration process. However, some low-expressing T_0 plants were included, to determine a minimum value of PAT protein required to confer herbicide resistance.

Milestone #7: Possible initiation of CDC-AFP (anti-fungal protein) transformation (Year 2).

Achievement: The anti-fungal proteins that were to be provided by AgrEvo Canada were not received.

Milestone #8: Initial T_4 CDC-*pat* plants tested in field for Liberty-tolerance and performance (Year 3).

Achievement: Insufficient seed from the CDC-*pat* plants was available for planting in the 1998 field season. Therefore, the homozygous T_5 seed from two transgenic 'Greenfeast' lines (PLP18 and DB131) was utilized for the first field trials. The test site for this transgenic trial was at the University of Saskatchewan's Kernen Crop Research Farm in an area designated for confined trials of transgenic plants. Permission for the field trial was granted by the Government of Canada, Canadian Food Inspection Agency, Plant Products Division, under application 98-PBI1-106-PEA. The plot consisted of randomized complete blocks with 4 replicates. Each replicate block contained one control and the two transgenic lines. The treatments consisted of four concentrations of Liberty® (0, 2, 4 and 8 l ha^{-1}, with 150g/l a.i.). A concentration of 8 l ha^{-1} represents more than twice the recommended dosage for Liberty®-tolerant canola. Each plot (2 m^2) contained 4 rows of 50 seeds spaced 20 cm apart, with 1 m spacing between plots. The rows were trimmed to 2.5 m, and the number of plants per plot was recorded. The plots were sprayed 32 d after planting. Individual plots were sprayed with a shrouded plot sprayer to minimize chemical drift. To confirm the presence of the *pat* gene in plants grown under field conditions, both PCR and Southern hybridization analyses were performed on leaf tissues of two random plants from each 0 l ha^{-1} plot. To estimate the level of *pat* gene expression, leaf tissue was collected from all four treatments of transgenic plants but only the 0 l ha^{-1} plots of untransformed 'Greenfeast' controls. The pods were harvested and the seed weights were determined for each plot. The concentration of PAT protein in the seeds was estimated from samples of 20 seeds, by ELISA assay as described above.

Milestone #9: Initial T_0 CDC-AFP plants recovered and tested (Year 3).
Achievement: See Milestone #7.

Milestone #10: Transformation of CDC-AFP plants completed (Year 3).
Achievement: See Milestone #7.

Milestone #11: Fourth generation of CDC-*pat* breeding material field tested for Liberty-tolerance and agronomic performance (Year 3).
Achievement: Cancellation of the project precluded field testing of the CDC-pat breeding material.

Results and conclusions

Since the aim of this project was the development of economic levels of herbicide tolerance in the field, only the results of the field testing will be presented in this final report.

In the field, the non-transformed control plants were severely damaged by even the lowest (2 l ha^{-1}) of Liberty herbicide. The growth of the transgenic lines was unaffected by spraying with the herbicide at levels up to 8 l ha^{-1}, more than two times the dose used for weed control in transgenic *Brassica napus* L. Small brown spots on the leaves of transgenic plants sprayed with both 4 and 8 l ha^{-1} Liberty®. A temporary discolouration or "bronzing" has been noted on transgenic Brassica napus sprayed with Liberty (Guide to Crop Protection 1999. Saskatchewan Agriculture and Food). Also, a temporary discolouration of transgenic tobacco and potato (De Greef *et al.* 1989.Bio/technology 7:61-64) observed after herbicide spraying, was attributed to surfactants present in the commercial formulation which, at higher herbicide concentrations, are more concentrated than in normal applications. The effect of herbicide dose was apparent in the control plots, when viewed 21 days after the herbicide spraying. In contrast, growth of the transgenic pea plants appeared uniform regardless of herbicide dose. The plots sprayed with 0 l ha^{-1} Liberty were easily identifiable by the presence of weeds, especially wild mustard. The location of each control plot could be identified from a distance in an overview of the field (Figure 3-1).

Figure 3-1. Overview of confined field trial 21 days after spraying. Individual plots of control (C) and transgenic (T) plants are identified, along with the concentration of herbicide (l ha^{-1}). Other blocks of control plants are easily identified by sparse coverage of the field.

In the field, the PAT protein in leaves of the two lines, DB131 and PLP18, was significantly different, at 686 and 627 pg PAT [µg total soluble protein]$^{-1}$, respectively. The two transgenic lines differed in the location of *pat* gene insertion; therefore, different responses to the field conditions could be expected. The effect of the herbicide dose was not significant. The concentration of PAT protein required for effective herbicide tolerance under field conditions has not been established. In tobacco, a low level of PAT (<0.01% of total soluble protein) was sufficient (De Greef *et al.* 1989.Bio/technology 7:61-64). In comparable terms, the two lines in the present study have levels between 0.07 and 0.08%.

Seed yields of control plants decreased dramatically with increased herbicide treatment in field plots. In contrast, seed yields of the two transgenic lines increased significantly as the herbicide dose increased (Figure 3-2). For the transgenic plants, seed yield of the 8 l ha^{-1} treatment was 21% greater than that of the 0 l ha^{-1} control.

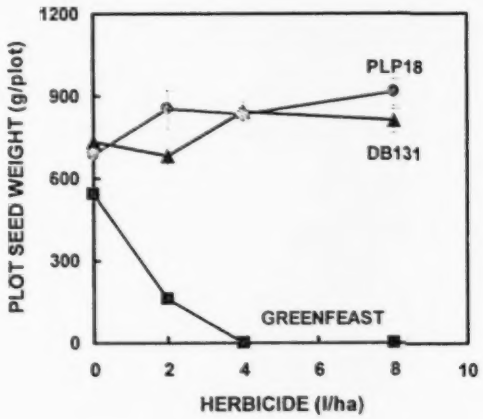


Figure 3-2. Mean final seed production for control (GF) and transgenic (DB131, PLP18) lines as affected by herbicide dose. Vertical bars represent the standard error of the mean.

The level of PAT protein present in the harvested (dry) seed of the Liberty-tolerant transgenic pea was measured to determine the amount that would be consumed, as part of an assessment of risks associated with transgenic peas. The level of PAT protein in transgenic seeds produced in the field (57 and 62 pg PAT [ug total soluble protein] $^{-1}$ for the two lines) was approximately 10% of that measured in the leaves. There was a highly significant difference in PAT protein content ($P<0.01$) among the plant lines, but the difference was only between the control 'Greenfeast' and the two transgenic lines. The analysis indicated that there was no significant effect of herbicide dose on PAT protein content ($P>0.73$).

The *pat* gene, inherited through six generations, was consistently expressed under field conditions. Although the amount of PAT protein produced was lower than under controlled-growth conditions, it was sufficient to confer herbicide tolerance to Liberty® at more than twice the dosage recommended for weed control in transgenic canola (Guide to Crop Protection 1999. Saskatchewan Agriculture and Food). In addition, there was no

evidence that the presence of the foreign gene and the protein produced had any detrimental effect on the agronomic performance of the transgenic plants. In control plots (0 l ha^{-1} Liberty), the transgenic plants yielded higher than control plants. Also, the transgenic plots sprayed with herbicide yielded significantly higher than those that did not receive the herbicide. Although a population of weeds was present at the time of harvest, early control by the herbicide apparently gave the peas sufficient time to get established. Production of a pea crop of commercial value would require the further development of homozygous lines of elite germplasm containing the *pat* gene. A paper prepared on the basis of these results will be submitted to Theoretical and Applied Genetics.

Sub-Project 4: New Genes for Inducing Systemic Disease Resistance
Drs. P. Fobert and C. Després

Background and Objectives

Systemic acquired resistance (SAR) is an effective, long-lasting and broad-range type of resistance that is naturally induced by localized infection with certain microbes. Under field conditions, SAR is effective at reducing disease and increasing yields. Preliminary results with peas suggest that SAR can control (>80% reduction in lesions) *Ascochyta pinodes*, the most serious fungal pathogen of peas in Saskatchewan. The specific objectives of this sub-project were to identify genes capable of inducing SAR, to produce transgenic pea germplasm expressing these genes and to assess their effectiveness.

Project activities related to milestones, achievements

Milestone #1: Inducing agents for pea systemic resistance identified (March 1999)

Achievement: Several genes with the potential of regulating the plant's ability to mount effective systemic disease resistance were identified as possible biological inducing agents. These included: NPR1, NPR1-related genes, TGA transcription factors and NIMIN-3. In addition, chemicals capable of inducing systemic resistance in other crops were also of interest. These included salicylic acid and benzothiadiazole (BTH).

Milestone #2: Level, extent and pathogen spectrum of inducing agents determined (March 00)

Achievement: Salicylic acid and BTH were not effective at inducing resistance to *Pseudomonas syringae* pv *pisi*. The rest of the inducing agents of interests were genes, accordingly it was not possible to directly test their ability to induce systemic resistance.

Milestone #3: Gene(s) for inducing stimulus identified (March 00) and characterized (March 01)

Achievement: The cDNA for the following genes were cloned: NPR1 and NPR1-related genes NPR2 through NPR5; TGA factors TGA1 through TGA7; and the NPR1 interacting gene NIMIN-3. Characterization of these genes included testing biochemical function and altering (increasing or decreasing) expression levels in transgenic plants to determine whether they induced pathogenesis-related (*PR*) gene expression and enhanced disease resistance. NPR1 had previously been shown to be a central regulator of systemic disease resistance. We were able to demonstrate that (a) NPR2 and NPR4 are required for full induction of *PR* genes as well as disease resistance; (b) TGA factors bind to important DNA sequences found in the promoter of *PR* genes and NPR1 enhances this binding; (c) in addition we have obtained evidence linking TGA factors in the regulation of stress responses.

Milestone #4: Transformation vector synthesis, initiate transgenic plant production (March 01)

Achievement: Binary T-DNA vectors containing all of the above genes under the control of strong constitutive promoters were constructed, and all vectors were used to produce transgenic Arabidopsis or tobacco plants for initial testing. Transformation vectors containing NPR1 under the control of the cauliflower mosaic virus 35S promoter (CaMV35S) as well as the tobacco constitutive promoter (tCUP) were used to produce transgenic peas (cultivars Greenfeast, Vienna or Mozart).

Milestone #5: Continued production of transgenic plants, assessment of effectiveness under laboratory conditions (March 02)

Achievement: More than 30 primary independent transgenic pea lines were produced. For lines expressing high levels of NPR1, material was carried through to the T_6 generation. Preliminary testing with *Ascochyta pinodes* indicates that the CaVM35S-NPR1 transgene confers enhanced protection under the assay conditions. Transgenic lines did not possess enhanced resistance to a virulent strain of *Pseudomonas syringae* pv *pisi*.

Results and conclusions

4.1 Screening for Systemic Acquired Resistance (SAR) Inducers:

SAR is associated with the expression of a specific set of genes. To facilitate our screen for agents capable of inducing SAR, we developed a novel strategy to allow us to rapidly detect expression of SAR-inducible genes. It consisted of creating gene fusions between the regulatory signals (i.e. promoters) from SAR-inducible genes and the protein coding region of an easily assayed reporter gene, β-glucuronidase (GUS). It was hypothesized that if the agent tested is capable of inducing SAR, transgenic plants containing the reporter gene construct would turn blue when stained with as GUS specific substrate, X-GLUC.

We produced a collection of transgenic pea plants containing GUS fused to two different SAR-inducible genes using *Agrobacterium*-mediated transformation. As summarized in our March 1999 progress report, none of the transgenic plants tested showed clear-cut induction of GUS activity after treatment with the SAR inducer salicylic acid (SA), or following infection with a bacterial pathogen (*Pseudomonas syringae* pv. *pisi*) and a fungal pathogen (*Ascochyta pinodes*). Two transgenic lines expressed GUS constitutively, while the other two failed to express GUS even after inducing treatments. In the absence of transgenic lines capable of expressing SAR-induced reporter genes, and because screening for inducing agents based on resistance to pathogens itself would be too costly and time-consuming, this approach was not pursued any further.

4.2 New candidate genes for inducing systemic disease resistance:

4.2.A. NPR1

Shortly after our proposal was submitted, a gene required for SAR was cloned from Arabidopsis[1,2]. Mutants in this gene are compromised in their ability to express *PR* genes and cannot establish SAR. The same gene was given two different names by independent groups; NPR1 (for non expressor of *PR* genes 1)[1] and NIM-1 (for no-immunity 1)[2]. We reasoned that overexpression of *NPR1* may increase SAR. A few other groups have now demonstrated that increasing levels of NPR1 enhances resistance to bacterial and oomycete pathogens in Arabidopsis, and a bacterial pathogen in rice[3-5].

To test the effectiveness of NPR1 in peas, we fused the coding region of the NPR1 cDNA to two strong constitutive promoters, the CaMV35S promoter and tCUP[6], as well as the light-inducible rbcS promoter from pea. These fusions were introduced into binary T-DNA transformation vectors (See Figure 4-1) and transformed into *Agrobacterium*. Strains containing these vectors were used to infect embryonic axes of three pea varieties ('Greenfeast', 'Vienna' and 'Mozart').

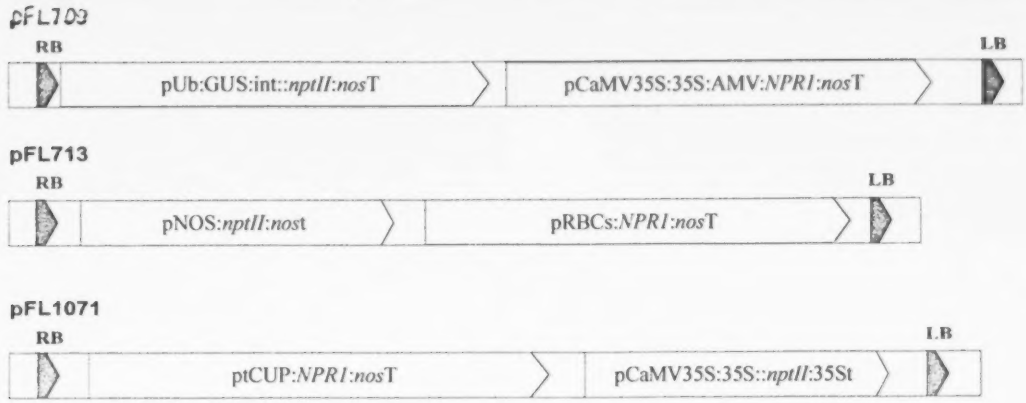


Figure 4-1. T-DNA transformation vectors used in this study. pUB, pea ubiquitin promoter; GUS:int:*nptII*, chimeric gene consisting of *E. coli β-glucuronidase* coding sequence containing an intron (int) fused in frame to the *neomycin phosphotransferase II* gene for kanamycin selection; pCaMV35S:35S:AMV, duplicated cauliflower mosaic virus 35S promoter along with alfalfa mosaic virus translational enhancer; *nos*T, *nopaline synthase* terminator; p*nos*, *nos* promoter; pRBCs, pea rubilose bisphosphate carboxylase small subunit promoter; ptCUP, tobacco constitutive promoter; 35St, CaMV35S terminator, RB, right T-DNA border repeat; LB, T-DNA left border repeat.

In all, more than 6,000 explants were infected, resulting in the production of numerous shoots and 30 independent transgenic (T_0) plants. Northern blot analysis of this material identified five individual lines expressing the transgene (see Figure 4-2 for representative result). In order to identify true-breeding lines, the T_0 material from several expressing lines was allowed to self-fertilize, and progeny (T_1) were analyzed for segregation of the transgene by northern and Southern blot hybridization. Using this approach, we produced and analyzed transgenic material expressing the transgene for up to 6 self-fertilized generations (T_6). As detailed in previous progress reports, we were not able to identify a true-breeding line which consistently expressed the transgene. In some cases, we were able to detect the presence of the transgene by Southern blot analysis, but could not demonstrate expression of the gene. We suspect that the transgene was silenced in these lines, a phenomenon which has previously been described in transgenic plants[7]. Our inability to identify true-breeding material caused a considerable delay in our efforts to test the material for interaction with pathogens.

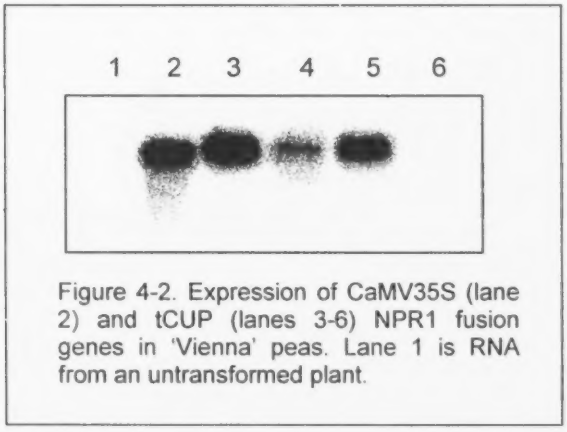


Figure 4-2. Expression of CaMV35S (lane 2) and tCUP (lanes 3-6) NPR1 fusion genes in 'Vienna' peas. Lane 1 is RNA from an untransformed plant.

Transgenic material expressing either the tCUP or CaMV35S fusions to NPR1 were tested for their response to two different pathogens. First, we tested the interaction with *Ascochyta pinodes* (a fungal pathogen) using a modified detached leaf assay. Given that all of the varieties used in this study were semi-leafless, we tested detached stipules. In order to describe disease symptoms, a disease index was developed (Table 4-1). Stipules from untransformed plants were used as control material.

Table 4-1. Disease symptom index. *A. pinodes* spores (10 µl of $2x10^5$ spores/ml) were inoculated onto detached pea stipules. The inoculated stipules were incubated in a box (15"L X 10"W X 9"H) on top of moist sterile gauze at 100% relative humidity. Disease symptoms were evaluated with a light dissection microscope after different times.

Disease Index	Description
0	No visible symptoms in the inoculated area under microscope
1	10% or less inoculated area shown necrotic lesion or flecks
2	10-30% inoculated area covered with necrotic lesion or flecks
3	30-50% inoculated area covered with necrotic lesion or flecks
4	50-80% inoculated area covered with necrotic lesion or flecks
5	Larger than 80% of inoculated area covered with necrotic flecks or lesion, the lesion becoming confluent, visible mycelium growth into adjacent area
6	Total inoculated area symptomatic with further development into adjacent area as concentric rings. Visible fungal growth and senescence in adjacent area

Results of the disease testing are presented in Table 4-2. Representative photos of disease symptoms are shown in Figure 4-3. Untransformed pea leaves (Vienna) displayed the highest disease symptoms (index of 4). Leaves from most of the plants transformed with the tCUP:NPR1 fusion showed similar disease symptoms to the untransformed leaves (6 of 8 leaves). This suggests that the transgene is not conferring substantial protection against this pathogen under the assay conditions. In contrast, most of the plants transformed with the CaMV35S:NPR1 fusion displayed reduced disease symptoms. For line CaMV35S-NPR1 B4, all 8 plants tested displayed disease symptoms ranging from 0 to 2 on the index. In most cases, less than 10% of the inoculated area contained necrotic lesions or flecks. With plant CaMV35S-NPR1 B15, 5 of the 8 plants displayed reduced symptoms, while 3 of the plants were comparable to the untransformed controls. These results suggest that transformation with the CaMV35S:NPR1 gene can confer enhanced protection against infection by *A. pinodes*. Additional testing will be required to confirm these results. These should include tests at the whole plant level, first in the greenhouse, and eventually under field conditions. However, silencing of the transgene in these lines will represent a problem for large-scale testing, as the presence of non-expressing individuals within the population will bias results. To ensure that plants are expressing the transgene, we have routinely analyzed the material by northern blotting. However, such analysis will be laborious to perform on a large scale.

To test whether overexpression of NPR1 could confer broad-host range disease resistance, we also inoculated stipules with the bacterial pathogen *Pseudomonas syringae* pathovar *pisi*. Material was infected as described in Lai *et al.*[8]. After 3 days, discs were cut from infected areas, ground in buffer, serially diluted, plated and the resulting colonies counted. Results presented in Figure 4-4 show that stipules from plants transformed with either tCUP:NPR1 or CaMV35S:NPR1 supported similar amount of bacterial growth as those from untransformed plants. This suggests that the NPR1 transgene is not conferring any protection against this pathogen.

Table 4-2. Disease symptoms in pea plants transformed with NPR1 transgene.

Plant	Disease Index
Untransformed -a	4
Untransformed – b	4
tCUP-NPR1 4/5 -1	1
-2	4
-3	4
-4	1
tCUP-NPR1 4/7 -1	3
-2	4
-3	4
-4	4

Plant	Disease Index
CaMV35-NPR1 B4 –1	1
-2	2
-3	2
-4	1
-5	0
-6	1
-7	1
-8	1
CaMV35-NPR1 B15 -1	1
-2	4
-3	4
-4	4
-5	1
-6	2
-7	1
-8	1

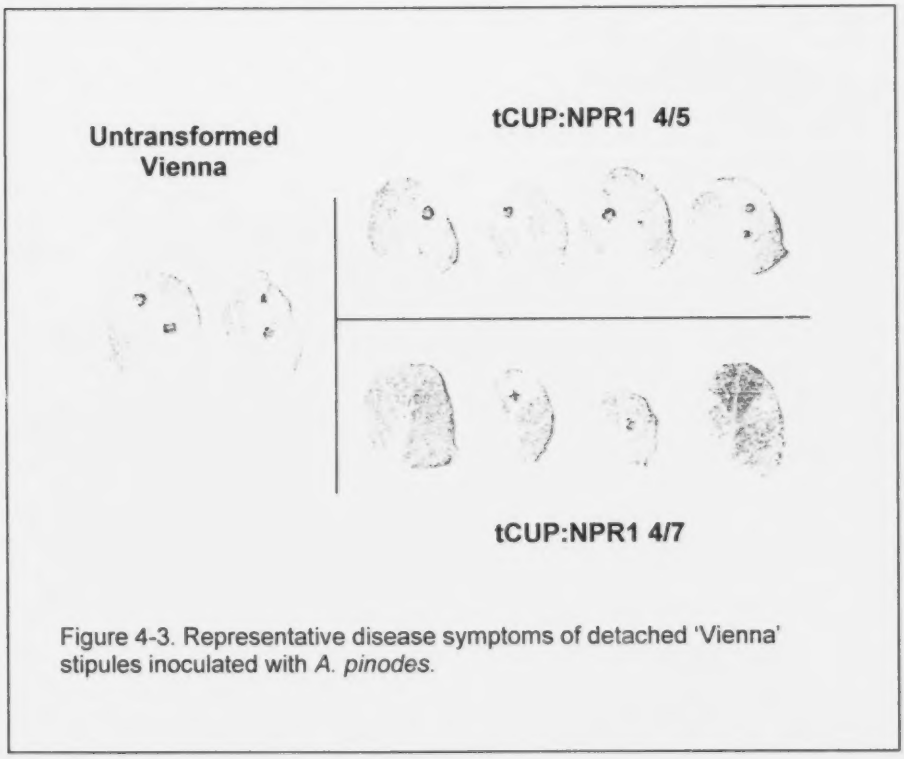


Figure 4-3. Representative disease symptoms of detached 'Vienna' stipules inoculated with *A. pinodes*.

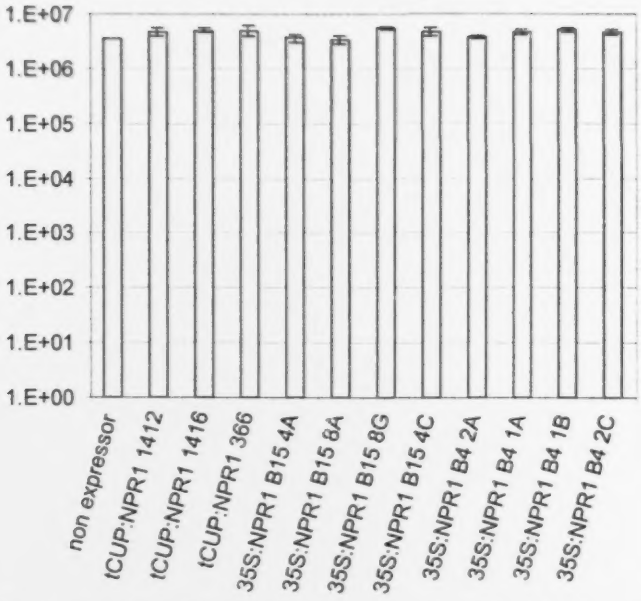


Figure 4-4. Average bacterial growth (colony forming units (cfu) per leaf disc) +/- standard error after 3 days in transgenic 'Vienna' peas inoculated with *Pseudomonas syringae* pv *pisi* at 10^6 cfu per ml.

Given the central role salicylic acid (SA) plays a key role in disease resistance associated with NPR1[9], we decided to further explore the role of this metabolite in the interaction between peas and *P.syringae* pv *pisi*. In a number of species, application of SA or SA analogs can confer enhanced resistance to *P. syringae*. Conversely, blocking SA synthesis using the bacterial gene *salicylate hydroxylase* (Nah G) can compromise resistance to this pathogen. As shown in Figure 4-5 spraying pea plants with SA or the SA analog BTH did not confer any additional resistance to *P. syringae* pv *pisi*, nor did expression of Nah G compromise resistance. Taken together our data indicate that neither NPR1 nor SA play important roles in determining the outcome of the interaction between peas and *P. syringae* pv *pisi*.

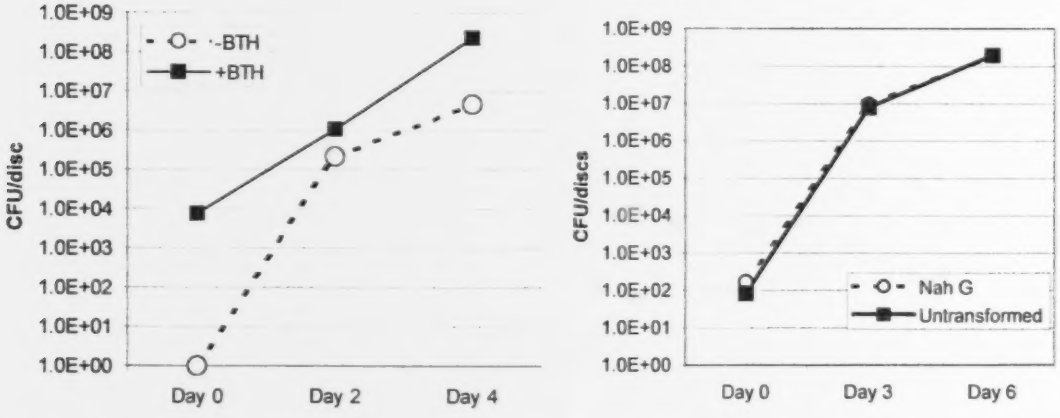


Figure 4-5. Role of salicylic acid during the interaction between peas and *P. syringae*. In the left graph, plants were treated with water or the SAR inducing chemical BTH. Such treatment actually enhanced susceptibility to this bacterial pathogen. In the right graph, untransformed peas ('Greenfeast') and transgenic plants expressing the Nah G gene were inoculated with *P. syringae*. Reduction of SA in this plants did not result in enhanced susceptibility to this bacterial pathogen.

4.2.B. Genes Encoding Proteins that Interact with NPR1

As part of the PBI core program, we identified two groups of proteins that interacted with NPR1, and cloned their genes. One group of proteins identified belongs to a family of transcription factors called TGA factors, because they bind to the DNA sequence "TGAC". Transcription factors act as molecular switches, controlling the expression of genes. Members of TGA family have been shown to bind to a SA-inducible promoter element known as *as-1*[10]. Given the central role of SA in triggering SAR, we believed that the interaction between NPR1 and TGA factors might be important in regulating gene expression leading to SAR. Accordingly we assessed whether these genes may represent effective inducers of disease resistance when expressed in transgenic plants. Given that the genes are already "cloned", this avenue of research had the potential to save a considerable amount of time.

We determined that at least one function of NPR1 was to enhance the ability of TGA factors to bind to cognate promoter elements[11] (see also progress report of March, 1999). We also demonstrated that TGA factors could bind to promoter elements required for the SA induction of *PR* genes and NPR1 enhanced this binding. Taken together, our results – along with published information from other groups – are consistent with a model whereby pathogen challenge leads to SA accumulation, which activates NPR1 by some yet uncharacterized mechanism. The activated NPR1 then interacts with TGA factors to enhance their binding to elements of the *PR* gene promoters, thus activating *PR* gene expression and the establishment of disease resistance (see Figure 4-6).

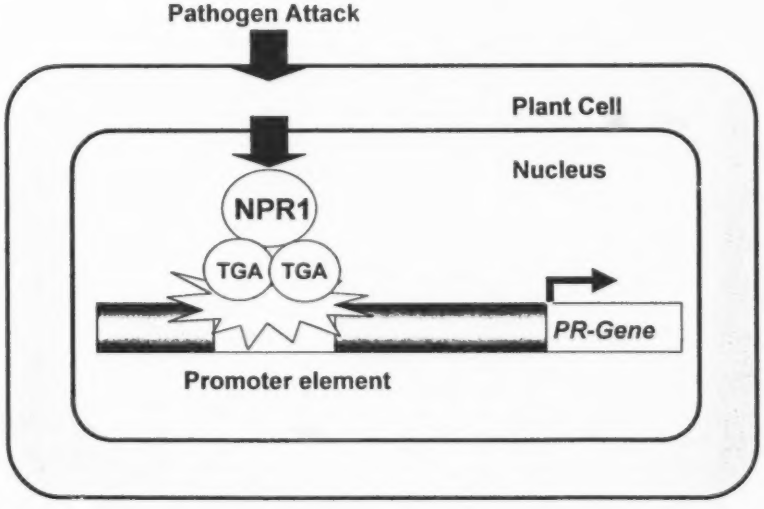


Figure 4-6. Model for NPR1 function in regulating *PR* genes

Based on this model, it may be possible to engineer disease resistance by increasing the binding of NPR1 to TGA factors, or enhancing TGA binding to *PR* gene promoters. We have now identified a domain in NPR1 that is essential for enhancing the DNA

binding ability of TGA factors. We have produced transgenic plants expressing a derivative of NPR1 that contains a mutation in this domain and found these to have enhanced susceptibility to the bacterial pathogen *P. syringae* pv *tomato* (Figure 4-7). This provides a link between the NPR1-mediated enhanced binding of TGA factors to *PR* gene promoters and disease resistance. Also, we have defined amino acids within TGA factors required to interact with NPR1. Of note, by altering key amino acids, it is not only possible to disrupt the interaction between NPR1 and a TGA factor, but it is also possible to create new interactors from factors that normally do not bind to NPR1. Patent protection for this novel switching mechanism is being sought[12]. We have also produced a number of transgenic lines that overexpress various TGA factors, but have not had the opportunity to analyze these. As part of this work, we also identified a new member of the TGA family (TGA7) and performed preliminary characterization.

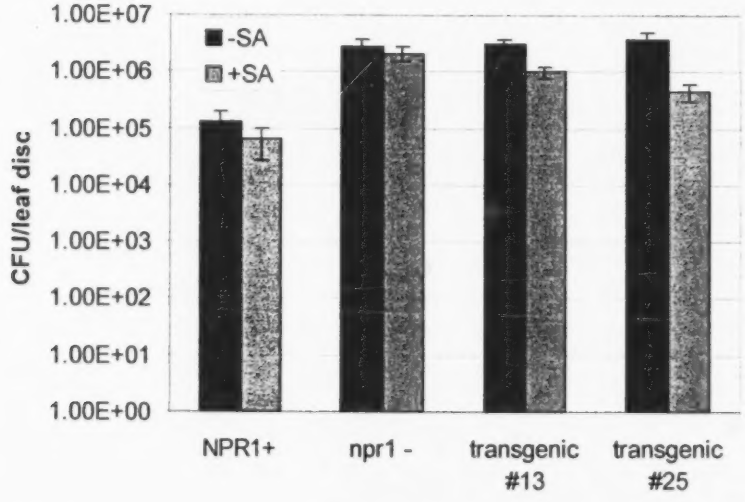


Figure 4-7. Transgenic plants expressing a mutant version of NPR1 affected in its ability to enhance the binding of TGA factor to promoter elements are highly sensitive to infection by *P. syringae*. Data represent the average of 4 to 10 independent samples +/- standard error. NPR1+, wild type plants; npr1-, mutant plants lacking NPR1 function and unable to mount SAR; transgenic #13 and #25, two independent transgenic lines overexpressing the mutant version of NPR1. Both transgenics were in the npr1- genetic background. In the absence of SA, these transgenic lines support on average 32 X more bacterial growth than the NPR1+ plants.

4.2.C. NPR1-Related Genes

Plant genomes encode for a small family of NPR1-related genes. None of these have been functionally characterized. However, given the central role that NPR1 plays in SAR resistance, it is plausible that at least some members of this gene family may also function as effective inducers of SAR. We first cloned 4 genes related to NPR1 and called these NPR2, NPR3, NPR4 and NPR5 and found that NPR2-4 are capable of interacting with TGA factors (see progress report October, 1999).

The functions of NPR1 and other members of the family cannot be the exactly the same. This is because mutation of *NPR1* leads to an unambiguous phenotype (disease susceptibility), even in the presence of presumably functional alleles of the remaining genes. To assess the function of these genes, we first overexpressed them in transgenic

plants. These plants have only been characterized in a preliminary fashion. However, it appears that plants overexpressing NPR2 may be able to constitutively express *PR* genes. We must now perform disease testing on these plants, as well as those overexpressing NPR3 and NPR4. As a second means of determining whether these genes participated in disease resistance, we used reverse genetics to isolate mutations in each of NPR2, NPR3 and NPR4. Results indicate that these mutants are all hypersusceptible to *P. syringae* infection (see Figure 4-8), clearly establishing a role of the corresponding genes in disease resistance. The *npr2* and *npr4* mutants are also compromised in their ability to express certain *PR* genes, although the individual profiles of *PR* gene are distinct from *npr1*. We have combined the new mutants with the *npr1* mutant. Preliminary results indicate that at least one double mutant combination is more susceptible than either single mutant, indicating a possible additive or synergistic gene interaction. We are now creating transgenic plants expressing different combinations of NPR1, NPR2, NPR3 and NPR4 to test whether pyramiding two or more of these genes will lead to greater disease resistance than overexpression of either alone.

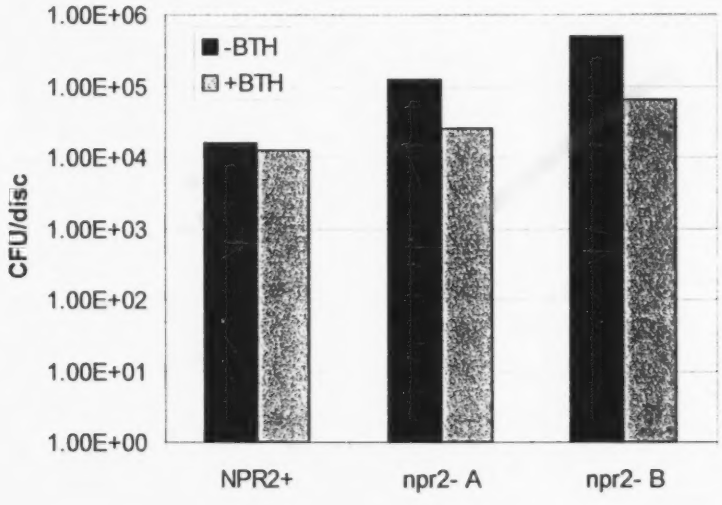


Figure 4-8. NPR2 mutants are hypersusceptible to infection by *P. syringae*. Two separate lines (npr2- A and –B) were tested in the presence or absence of the SAR inducer BTH.

In conclusion, we have achieved the key deliverable and performance indicators as outlined in Appendix A of the contract for this subproject. Namely, we have isolated at least one *bone fide* SAR inducer gene (NPR1) and at 3 or more additional genes (e.g. NPR2, NPR3 and NPR4) which show promise as inducer genes. We have constructed transformation vectors containing these inducer genes and produced transgenic pea germplasm that expresses NPR1. Preliminary results indicate that this germplasm has enhanced tolerance to a fungal pathogen (*A. pinodes*) under controlled laboratory conditions.

4.3 References:

1. **Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X.** (1997). The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88**, 57-63.
2. **Ryals, J.A., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.Y., Johnson, J., Delaney, T.P., Jesse, T., Vos, P., and Uknes, S.** (1997). The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor IκB. Plant Cell **9**, 425-439.
3. **Cao, H., Li, X., and Dong, X.** (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl. Acad. Sci. USA **95**, 6531-6536.
4. **Friedrich, L., Lawton, K., Dietrich, R., Willits, M., Cade, R., and Ryals, J.** (2001). NIM1 overexpression in Arabidopsis potentiates plant disease resistance and results in enhanced effectiveness of fungicides. Mol. Plant-Microbe Interact. **14,** 1114–1124.
5. **Chern, M.S., Fitzgerald, H.A., Yadav, R.C., Canlas, P.E., Dong, X., and Ronald, P.C.** (2001). Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. Plant J. **27,** 101–113.
6. **Foster, E. Hattori, J., H. Labbe, T. Ouellet, P.R. Fobert, L.E. James, V.N. Iyer, and Miki, B.L.** (1999) A tobacco constitutive promoter, tCUP, revealed by T-DNA tagging. Plant Molecular Biology **41,** 45-55.
7. **Matzke, M.A., Mette, M.F., and Matzke, A.J.** (2000) Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. Plant Mol. Biol.**43,** 401-15.
8. **Lai, F.-M., DeLong, C., Mei, K., Wignes, T. and Fobert, P.R.** (2002) Analysis of the DRR230 family of pea defensins: gene expression pattern and evidence of broad host-range antifungal activity. Plant Science **163,** 855-864.
9. **Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gutrella, M., Kessmann, H., Ward, E., and Ryals, J.** (1994). A central role of salicylic acid in plant disease resistance. Science **266**, 1247-1250.
10. **Xiang, C., Miao, Z.-H., and Lam, E.** (1996). Coordinated activation of *as-1*-type elements and a tobacco glutathione *S*-transferase gene by auxins, salicylic acid, methyl-jasmonate and hydrogen peroxide. Plant Mol. Biol. **32**, 415-426.
11. **C. Després, C. DeLong, S. Glaze, E. Liu, and P. R. Fobert**. (2000) The Arabidopsis NPR1/NIM1 Protein Enhances the DNA Binding Activity of a Subgroup of the TGA Family of bZIP Transcription Factors. Plant Cell **12,** 279-290.
12. **Després, C. and P.R. Fobert**. (2002) Redox switch regulation of plant transcription factors. Canadian Patent Application, June 2002.

Sub-Project 5: Field Assessment of Transgenic Pea Plants
Drs. J. D. Mahon, A. Vandenberg and P.L. Polowick

Background and Objectives

Prior to general introduction into any crop improvement program, it is essential to assess potential new transgenic crops under field conditions in order to ensure that the engineered genes are expressed without compromising plant performance. It is also prudent to determine the risk of uncontrolled spread of the introduced gene into surrounding fields via pollen distribution. In addition, comprehensive documentation of the biology of the species is a regulatory requirement prior to any commercialization of a new transgenic species. The specific objectives of this sub-project were to assess the stability and expression of genes in transgenic peas under field conditions, to assess the potential risk of outcrossing by transgenic peas growing in the field, and to develop a document on the biology of *Pisum sativum* L. for regulatory approval.

Project activities related to milestones, achievements

For this sub-project, the presented milestones, as written, were not conducive to an effective presentation of the work completed. Therefore, for this section, the achievements will be based broadly on the objectives of the project.

Milestone #1: Assess plant performance as well as the stability and expression of genes in transgenic peas under field conditions.

Achievement: This part of the project involved the production of homozygous lines of peas containing two separate vectors. The line PLP1 was recovered after co-cultivation with LBG66 (PBI3010), a binary plasmid with a *uidA*:*nptII* fusion gene, conferring both GUS reporter activity and resistance to the antibiotic kanamycin. This plant line consistently showed high expression of the *gus* gene. The line DB61 was recovered after co-cultivation with LBG71 (PBI3011), a binary plasmid with a *uid*A gene conferring GUS activity and a *pat* gene for resistance to L-phosphinothricin (L-PPT), the active ingredient in the herbicide Liberty®. This plant line consistently showed a low level of *gus* expression relative to the PLP1 line. Four separate homozygous lines with different expression levels were developed from DB61. Seeds from the T_4 and T_5 generations of the transgenic lines were used in field trials in 1997 and 1998, respectively. Each year, six pea lines including the PLP1 line, four DB61 lines and a control 'Greenfeast' line were planted in 2-row plots in a randomized block design with 4 replications. GUS activity was assessed in the tissues of leaves at three stages of leaf development. During reproductive development similar assays were performed on one leaf, the young pod wall and developing seeds. At harvest, three plants were sampled from each plot. For each of these plants, the dry weight of empty pods, combined stems/leaves, and seeds, as well as the numbers of pods and seeds was recorded. For the remaining plants in each plot, the numbers remaining and the total seed weight were recorded. Data were analyzed using the GLM Procedure of SAS for the tests of hypotheses for mixed model analysis of variance (SAS Institute). Variation among treatment means was analyzed using the LSD test at $P \leq 0.05$.

Milestone #2: To assess the potential risk of outcrossing by transgenic peas growing in the field.

Achievement: For this field trial, a central plot with four 15 m rows of transgenic pea line PLP1 (T_5, as described above) was planted on a north-south axis, perpendicular to the prevailing westerly wind. The PLP1 line was in the 'Greenfeast' background and, as such, had normal leaf morphology. Four blocks, containing 3 'trap' varieties, were seeded in random order on each side of the central transgenic plot and at right angles to it. Each of the 48 'trap' plots consisted of four rows with 1 m spacing between cultivars. All three 'trap' cultivars 'Montana', 'Carneval' and 'Tipu' had a recessive semi-leafless trait and lacked the *gus*A gene. The three cultivars were chosen for different flowering times to ensure a maximum overlap of flowering with that of the transgenic plants. At the end of the growing season, all pods were collected from the 48 'trap' plots and seed was stored for further analysis. From each plot, a random sampling of 100 seeds was planted and maintained in a controlled growth chamber for 10 to 14 days. A disc (4-mm dia.) was removed from a stipule of 96 plants from each of the 48 plots. The discs were placed singly into 200 µl of buffer containing X-Gluc solution with Tween-80 (0.1 %) in 96-well microtitre plates. The discs were incubated at 37°C for 48 h, after which they were cleared with 70% ethanol and scored for blue pigment. The same plants were scored for the presence of normal leaves and both traits were expressed as a percentage of the plants assayed. Any offspring of the 'trap' plants that result from pollination by the transgenic PLP1 plants would have normal leaves and would exhibit *gus* activity, as both are genetically dominant factors. The experiment was repeated in 1999 with T_6 seed and the same directional layout, at a different location.

Milestone #3: To develop a document on the biology of *Pisum sativum* L. for regulatory approval.

Achievement: The original project submission for this project included the preparation of a 'regulatory document' including a description of pea biology. This document was proposed to satisfy one of the requirements for the commercial release of herbicide tolerant peas from Sub-Project #3. With the cancellation of this project, the document was no longer considered necessary.

Results and conclusions

Field assessment of gene expression and growth performance of transgenic peas

The development of homozygous lines of PLP1 and DB61 through the fifth generation confirmed that inheritance of the inserted genes was stable. In addition, consistent and continued expression in the field was observed through both the vegetative and reproductive stages.

In order to accommodate the divergent values between the control plants and those with a high *gus* activity, the mean *gus* activity of both the vegetative and reproductive stages of both years of the test are presented on a $\log_{10}$ scale (Figure 5-1). There was no significant difference based on the year, the block, or which leaf (first, third, or fifth) was

tested during the vegetative phase (individual data not shown). Similarly, there were no significant differences between the tissues tested during the reproductive phase of growth (leaf, pod, seed). The analysis revealed that the only significant differences ($P<0.0001$) were between the plant lines. As in controlled environment conditions used for the development of the homozygous lines, the lowest *gus* activity in the field was observed in the untransformed 'Greenfeast' (GF) plants, with the highest activity in line PLP1. Consistent and occasionally significant differences in *gus* activity among the four DB61 lines continued even though they were developed from the same transformation event. While the genetic source of this variability has not been determined, it seems possible that segregation of plant genes, other than the *gus* gene, could alter the expression of the *gus* gene. The three distinct levels of *gus* expression (control, DB61, PLP1) provided a good comparison to test if a metabolic load can be associated with the production of an extra, foreign protein.

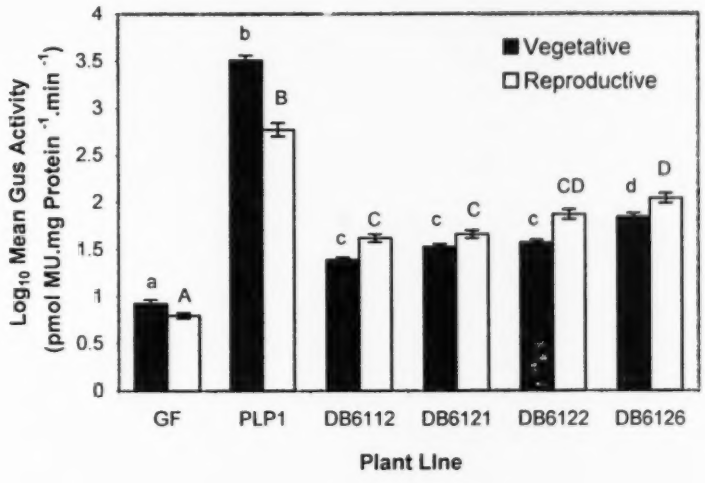


Figure 5-1. Mean *gus* activity of six lines of *Pisum sativum* plants during vegetative and reproductive growth from two years of field trials. The vertical bars represent the standard error of the mean (n=144)

* Bars with the same letter and case are not significantly different, based on a t Test (LSD) ($p \leq 0.05$)

At the time of harvest, data was collected on the basis of individual plants as well as on the plots. Statistical analyses indicated that there was no significant difference between the two years of the field trial or between the blocks; therefo.e, the data for the mean of the total plant weights for three plants from each block (n=144) is pooled (Table1). Overall, the total weight per plant appears to be greater in the control (GF) plants than in the other lines; this may reflect the decreased plant density caused by poor germination of the control plants (Table 5-1). The resulting reduced plant density would allow each plant more space for development with less competition for light and moisture. However, statistical analysis showed that the differences in the total plant weight between the plant lines were not significant.

Table 5-1. Seed germination and final harvest characteristics

Plant Line	Total Plant Weight (g) (n=24)	Seed Weight /Plant (g) (n=24)	Total plot yield (g) (n=8)	Number of emerged plants/row (n=8)
GF	36.78 (3.24)	15.56 (1.62)	242.25 (18.72)	11.88 (1.57)[a]
PLP1	29.70 (3.06)	12.55 (1.63)	317.01 (26.83)	18.50 (1.13)[b]
DB61-12	26.28 (2.17)	9.09 (0.99)	268.43 (20.33)	20.13 (0.85)[b,c]
DB61-21	26.92 (2.48)	11.96 (1.52)	340.41 (24.72)	19.88 (1.06)[b,c]
DB61-22	25.97 (2.38)	10.33 (1.44)	301.14 (26.26)	20.13 (1.20)[b,c]
DB61-26	24.80 (2.29)	9.82 (1.32)	266.41 (23.73)	22.25 (0.77)[c]

Mean values are followed by the standard error of the mean in parentheses
In last column, values followed by the same letter are not significantly different

The year and block also did not significantly influence the seed weight per plant; therefore, this data has been pooled for presentation (Table 1). As with the total weight per plant, the control plants appear to have a higher yield than the transgenics. However, these differences were not statistically significant.

The total yield per plot was lowest for the control (GF) line, reflecting the lower plant density. However, this difference was not statistically significant. Data on pod weight, pod number, seed number, stem plus leaf weight and the number of seeds per pod are also available but will not be presented here.

If there was a metabolic cost to the plant for production of the β-glucuronidase protein, it would be expected to have a greatest in the high-expressing PLP1 line. From this data, it does not appear that the production of the additional protein, even in high concentration, provides a detrimental load to the transgenic plants. Differences between the lines in terms of individual plant weight, seed weight per plant and per plot, were more a reflection of seed germination and plant density per plot. The poor germination of the control (GF) seed was surprising as it was produced under the same conditions and at the same time as the transgenic seed. A manuscript based on this data is in preparation.

Field assessment of outcrossing from transgenic pea plants

Table 5-2 shows the frequency of recovering plants with normal leaf forms and/or GUS activity from the two year study. Plants grown from the seed of 'Tipu' plots displayed a high frequency of the dominant normal leaf form; however, the normal leaf form was only rarely associated with the GUS activity expected from cross-pollination with transgenic (PLP1) pollen. Southern hybridization analysis of 'Tipu' offspring with normal leaf form but no GUS activity ruled out the possibility that the *gus*A gene was present but not functioning. Testing a sample of 'Tipu' seed used in the field in 1999 indicated that the high frequency of normal leaves was caused by heterogeneity of the seed source. Consequently, frequency estimates based strictly on leaf form were considered to be unreliable, especially for 'Tipu'. Thus, only progeny with both dominant traits were considered to be the result of an outcrossing event.

There was no detectable GUS activity in any plants assessed from offspring of the 1997 field trial (Table 5-1). However, in the 1999 trial, GUS activity was detected in the offspring of plants from two plots of 'Montana' and one plot of 'Tipu'. These three plots were all located in close proximity to the transgenic donor plants. In each case (five plants in total), both dominant markers were present. There were no cases in which a

semi-leafless plant had GUS activity. Overall, the mean frequency of outcrossing over the two year trial was 0.07% ('Montana', 0.11%; 'Tipu'. 0.09%; 'Carneval', 0.00%). Despite prevailing winds and the observed presence of insects as potential cross-pollinators, all of the recorded incidences of cross-pollination were in the 'trap' plots closest to the transgenic line.

Table 2. Summary of transgene recovery in three pea cultivars.

Year	Cultivar	Percent with Normal leaf	Percent with Gus activity
1997	Montana	0.00 (0.00)[1]	0.00 (0.00)
	Carneval	0.65 (0.37)	0.00 (0.00)
	Tipu	5.13 (0.96)	0.00 (0.00)
1999	Montana	0.21 (0.15)	0.21 (0.15)
	Carneval	0.20 (0.14)	0.00 (0.00)
	Tipu	15.78 (2.01)	0.18 (0.18)

[1]Number in parentheses represents the standard error of the mean

The current results provide a clear demonstration that transgenes can be transferred within the *Pisum sativum* species. However, their frequency of recovery in 'trap' plants is very low (mean = 0.07%), with no cases observed in 1997. The results also show that, in peas, the trangenes do not travel very far from their source. This would indicate that a smaller isolation distance would be required for pea relative to those recommended for other crops such as corn or *Brassica*.

A paper on this subject has been published in *Transgenic Research* and a copy is attached.

Sub-Project 8: Transposon Mutagenesis for Genetic Improvement of Pulse Crops
Dr. J. D. Mahon, Dr. P. L. Polowick, Ms. M. Anderson and Mr. D. Baliski

Background and Objectives

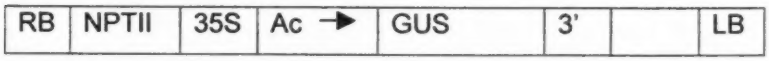
Transposon mutagenesis is a biotechnology tool for identifying and characterizing plant genes. This system combines the ability of mutagenesis to alter plant genes with a molecular 'tagging' system that can be used to recover genes that control structural, metabolic and regulatory aspects of plant function. Because pulse crops are similar, a beneficial gene found in one species may be equally valuable when transformed into other pulses. Thus, transposon technology can produce genetic variability for plant breeding as well as new proprietary genes (IP) that can be modified and incorporated into other crops. Although transposons ('jumping' genes) are found in pea, they have not been well characterized. The Ac/Ds transposon system from corn has been shown to function in several species including the legumes soybean and *Lotus japonicus*.

For plant breeding, the effectiveness of any kind of mutagenesis depends on the frequency of mutant production and the ability to identify traits that can improve crops. For biotechnology, mutation frequency is also important, but so is the ability to recover the controlling gene sequences. This proposal is for a "Proof of Concept" project that would introduce a corn transposon into peas, assess the frequencies of transposon excision and mutation and examine the possibilities for recovering of mutant genes.

Project activities related to milestones, achievements

Milestone #1: Transgenic peas produced with Phase 1 gene constructs.
Achievement: Two transposon constructs (Figure 8-1), obtained from Dr. Judith Strommer of the University of Guelph, were introduced into the EHA105 strain of *Agrobacterium tumefaciens* and used for transformation into pea plants. A total of 71 transformed peas, from 22 independent transformation events, were recovered after co-cultivation with pAcG43-1B3 (LBG100), using hygromycin for chemical selection. The other Phase 1 *Agrobacterium* vector, pAcG-1B3 (LBG98), was less effective, with a total of only 24 plants from 10 independent events recovered. Two new pairs of primers for PCR (polymerase chain reaction) analysis were developed to confirm the presence of sequence from the hygromycin phosphotransferase gene and the *Ac* transposon in the T_0 plants and to test for segregation of these genes in subsequent generations.

pAcG-1B3 (LBG98)



pAcG43-1B3 (LBG100)

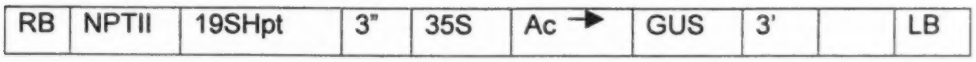


Figure 8-1. Constructs containing the corn transposon (*Ac*) have the neomycin phosphotransferase (*npt*II) gene for kanamycin resistance. pAcG43-1B3 also has the hygromycin phosphotransferase (Hpt) gene for hygromycin resistance. The Ac element separates the ß-glucuronidase (*gus*) gene from the 35S promoter. RB = right border; LB = left border.

Milestone #2: T_0 plants examined for visible mutation and GUS expression.
Achievement: The initial (T_0) plants were regularly checked for abnormalities throughout all stages of development. With these constructs, GUS activity can only occur if the transposon 'jumps' allowing the fusion of the *gus* gene to the promoter. Leaf discs (8 mm dia) removed from T_0 plants were incubated with an X-Gluc solution. If the transposition event only involves a sector of the plant, it will only be detected if that sector is sampled. T_1 seedlings (4-5 expanded leaves) were sacrificed and completely submerged in an X-Gluc solution to detect all events at that stage; the amount of X-Gluc solution needed precluded the submersion of larger plants.

Milestone #3: Corn regulatory constructs acquired and Phase 2 constructs produced.
Achievement: The use of anthocyanin pigmentation was proposed as an alternative reporter system permitting detection without the destruction of plant tissue. Two regulatory genes from corn, *R-S* and *Lc*, were obtained from Dr. Karen Cone (University of Missouri) and Dr. Susan Wessler (University of Georgia), respectively. Each of the genes was introduced with different orientations and promoters to create 10 plasmids (LBG101-LBG110), lacking the *Ac* transposon, which were transformed into peas (cv. Mozart). Subsequently, four constructs were produced containing the *Ac* transposon with one of the two regulatory genes (*Lc*, *R-S*) involved in anthocyanin production, replacing the *gus* gene used in Phase 1 (Figure 8-2).

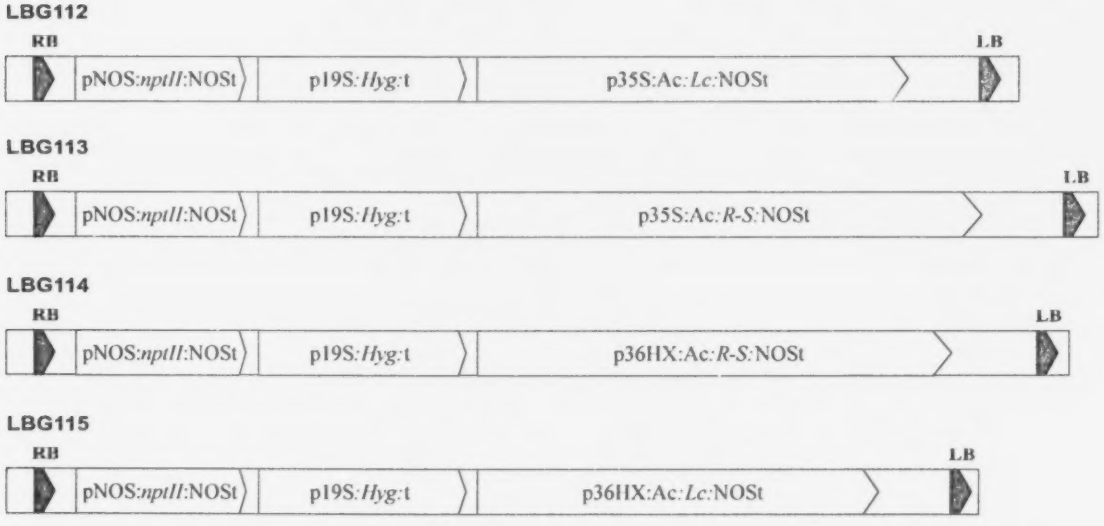


Figure 8-2. Four new vectors produced containing a regulatory gene from corn (*Lc*, or *R-S*), with one of two promoters, 35S or the polyubiquitin promoter 36HX.

Milestone #4: Transgenic peas produced with Phase 2 constructs.
Achievement: A total of 132 plants from 42 independent events were recovered after transformation with the 10 initial constructs containing the regulatory genes for anthocyanin production but without the *Ac* transposon. Using the four vectors (LBG 112, 113, 114, 115, Figure 8-2) with an *Ac*

transposon between the promoter and the regulatory gene (replacing the GUS gene used in Phase 1), a population of T_0 plants was recovered (Table 8-1). The presence of the regulatory genes was confirmed using new PCR primers developed to detect segments of the homologous *R-S* and *Lc* genes.

Table 8-1. Transgenic pea plants recovered after co-cultivation with *Agrobacterium* vectors containing the *Ac* transposon from maize and corn regulatory elements (*R-S*, *Lc*) for anthocyanin production.

***Agrobacterium* vector**	**# of independent events**	**Total # of rooted plants recovered**
LBG112	33	97
LBG113	13	42
LBG114	4	8
LBG115	7	35

Milestone #5: T_0 plants from Phase 2 examined for visible mutation and anthocyanin production.

Achievement: As with Milestone #2, the T_0 plants (plus subsequent generations) were regularly checked for abnormalities throughout all stages of development. With the anthocyanin markers, it was not necessary to sacrifice material in order to look for evidence of transposon excision.

Milestone #6: Assessment of transposon excision using callus from T_1 plants.

Achievement: To avoid using the entire population of T_1 seed for callus production, this part of the project was delayed until a larger supply of T_2 seed was available. Following procedures previously developed in the laboratory, callus cultures were established from the young roots, stems and leaves of seeds germinated under sterile conditions.

Milestone #7: Assessment of transposon insertion in genome with Southern blot.

Achievement: Southern hybridization analysis indicates the number of insertions of the introduced genes into the transgenic plants. Southern hybridization analysis of each independent event, including most of the clones, was performed using the restriction enzyme *BamH*1 and probes for fragments of the *Ac* transposon, the anthocyanin regulatory genes (*Lc*, *R-S*), and the hygromycin phosphotransferase gene (selectable marker).

Milestone #8: Estimates of transposon excision and re-insertion frequencies.

Achievement: With plants containing the *Ac*, Southern hybridization analysis can also determine if the transposable element has 'jumped' and reinserted. With *Agrobacterium* vectors LBG112 and 115, plant DNA cut with the restriction enzyme *BamH*1 should show the same banding pattern when probed with either the *Ac* probe or a probe for the anthocyanin regulatory genes. Any differences reveal excision and reinsertion, even where no morphological changed is evident. With the other *Agrobacterium* vectors (LBG100, 113, 114), *BamH*1 fragments of a specific size are expected if the transposon and the reporter gene are still associated. In subsequent generations, differences between banding patterns between the parents and offspring or between siblings suggest movement of the transposon.

Retention of these changes to the next generation is an indication that the event has been carried through the germline.

Milestone #9: Estimates of mutagenesis frequency.
Achievement: Within the scope of the present study, only mutations which caused visible, morphological changes were considered. All of the transposon-containing transgenic plants from each generation were regularly examined for abnormalities; these included leaf size and shape and pigmentation (variegation), branching patterns, fertility (male and female).

Milestone #10: Assessment of anthocyanin-based reporter system.
Achievement: The development of anthocyanin pigmentation was easy to follow because it did not require any treatment or the destruction of tissue. Starting at the time of co-cultivation, the explants and resulting plants were regularly examined for red colouration. This was compared to results from Southern hybridization analysis using fragments of the *R*-S and *Lc* genes as probes and PCR analysis using primers developed for these corn regulatory genes.

Milestone #11: Recovery of mutant gene.
Achievement: Large samples of DNA were extracted from plants with abnormalities of interest. DNA fragments of an appropriate size were identified using various restriction enzymes and Southern blotting with part of the *Ac* transposon as a probe. The eluted bands were then self-ligated to circularize the DNA. Using primers designed to match the transposon, but in a reverse orientation, inverse PCR was used to amplify the DNA flanking the *Ac* transposon. The resulting fragments were sent for sequencing and BLAST searches were performed to compare the results to known gene sequences.

Results and conclusions

During the period of study, it was shown that the *Ac* transposable element from maize functions in peas. There was evidence of excision and re-insertion, as well as morphological mutations in the transgenic plants recovered after co-cultivation with *Agrobacterium* vectors containing the transposable element.

With the Phase 1 vectors used in this study, GUS activity can only occur if the transposon 'jumps' allowing the fusion of the *gus* gene to the promoter. Sectors of GUS activity (Figure 8-3a) were detected by histochemical staining of leaf discs (8 mm dia) from 4 of 21 tested plants using the appropriate GUS substrate (X-Gluc). Two additional plants had detectable GUS activity, as measured by quantitative MUG analysis. If movement of the transposon were rare, activity would not be expected in so many randomly selected samples. When entire plants were submerged in X-Gluc solution, sectors of blue were observed on leaflets, stipules, stems (Figure 8-3b), roots (Figure 8-3c), and nodules. Without destroying the tissues of interest, it was impossible to follow any enlargement of these sectors.

Figure 8-3. Evidence of transposon jumping; GUS activity revealed by X-Gluc assay of a) a leaf disc; b) a stem from and entire submerged T_1 plant; c) roots of a submerged T_1 plant.

The most commonly-observed abnormality was that of variegation or loss of chlorophyll colouration in the shoot (Figure 8-4 a,b). This was observed in small sectors or through as many as eight continuous nodes but did not appear to pass through the germ line. In cases where the tissue was completely white, the shoot died back prior to producing any seed.

Figure 8-4. Examples of variegation observed on plants containing the *Ac* transposon; a) portion of leaflet from DB1086-1-5; Variegation on plant DB1081-3-1 includes the stipule (left), leaflets and tendril (right).

When using the anthocyanin-containing plasmids without the transposon, reddish cells, most prominent in areas of callus, were visible in the explants prior to the end of co-cultivation (Figure 8-5a). The red colour of the anthocyanins was generally not visible in green shoot tissues. However, two independent events (one with LBG 102 and one with LBG 110) produced plants with distinct anthocyanin patterns in specific areas; these included the base of the stipules (Figure 8-5b), on young tendrils, the pedicel of each flower, and the flower petals. A red/purple colour developed on the pods and darkened as they matured (Figure 8-5c). The resulting seeds were reddish brown and smaller than normal, but viable. This trait was inherited into the next generation; the seeds that were produced were normal in size and ranged in colour suggesting a gradient between those homozygous, hemizygous and negative for the anthocyanin trait (Figure 8-5d).
Using the *Agrobacterium* vectors containing both the *Ac* transposon and the regulatory genes for anthocyanin production, purple flowers developed on the T_0 plants of two independent events. There was no associated pigmentation at the base of the stipules, on the tendrils, nor on the pods. Transmission of the anthocyanin regulatory gene to subsequent generations, as determined by Southern hybridization and PCR analysis, was consistent with Mendelian inheritance of a single insertion event; however, the pigmentation of the flowers occurred on fewer plants than expected.

Aside from the T_0 plants which exhibited purple flowers, few examples of reddish pigmentation were observed in the plants containing the transposon. In two separate cases, purple sectors appeared on petals of plants in later generations. There was, however, no change in colour on any other plant tissue/organ. Also, in contrast to the

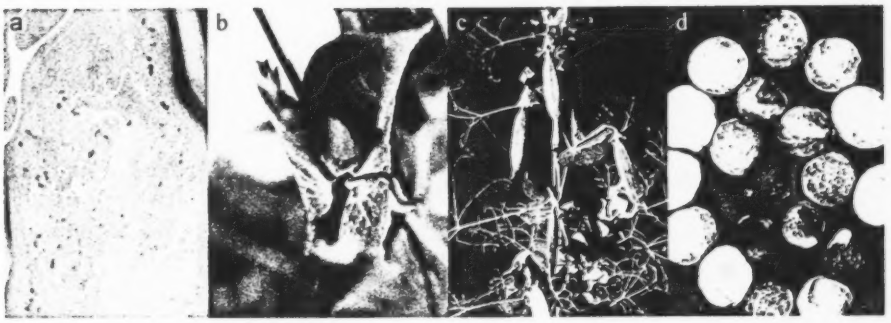


Figure 8-5. Anthocyanin production in pea tissues, as influenced by introduced corn regulatory genes: a) red spots on an explant 5 days after co-cultivation; b) red pigmentation at the base of stipules; c) red pods on maturing plants; d) T1 seeds from the same tranformation event in b) and c)

discrete spots of distinct red colouration observed on the original explants by the end of co-cultivation (Figure 4a), the callus developed a diffuse reddish-brown hue that was often indistinguishable from dead patches of cells. Since it was impossible to count 'jumping' events, this line of investigation was abandoned.

Although Southern hybridization confirmed the presence of the inserted corn regulatory elements for anthocyanin synthesis in the plants which produced red pigmentation, many plants with the genes (*R-S*, *Lc*) lacked the pigmentation. The anthocyanin-based reporter system did not reliably reveal the location of transposition, especially in green tissues. The exception was the production of small purple sectors in a few white petals. Therefore, despite the advantage of being non-destructive, this reporter system does not appear to be useful in peas.

Southern hybridization was used to confirm the presence of the *Ac* element, the reporter gene (*gus* or *Lc*/*R-S*) and the selectable marker (*hygromycin*) for each of the T_0 plants. As in previous studies in the laboratory at PBI, with the method of selection of putative transformants used, no escaped (untransformed) plants were identified. Southern hybridization analysis specified the number of copies of the introduced genes had been inserted. Also, with LBG112 and LBG115, comparison of the patterns obtained with the *Ac* probe and the anthocycanin (*Lc*, *R-S*) probe revealed events in which the *Ac* element 'jumped' and whether or not it reinserted elsewhere (Figure 8-6).

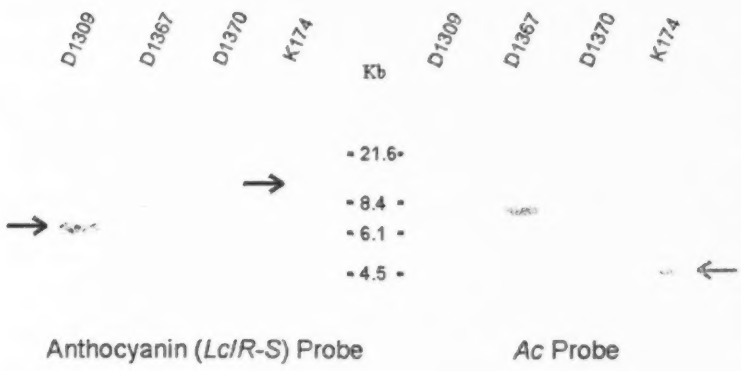


Figure 8-6. Southern analysis of DNA from four plants from co-cultivation with LBG112, probed with anthocyanin (left panel) and *Ac* fragments (right panel). Red arrows indicate bands in which the *Ac* and *Lc* genes became disassociated when the transposon jumped. The blue arrow indicates an *Ac* element which reinserted elsewhere in the genome.

It should be possible to determine the frequency of 'jumping' and reinsertion by following the offspring from one T_0 plant. In reality, it presents a complex pattern. For example, DB1319 was a plant recovered after co-cultivation with LBG112. It contained two copies of both the *Ac* element and the anthocyanin marker (*Lc*). The two probes gave different-sized bands, suggesting that both of the *Ac* elements had 'jumped" and reinserted. Of the 10 plants tested from the next (T_1) generation planted, both copies of the *Ac* remained in 40%, one 'jumped' and disappeared in the 30%, both were gone in 20% and one was gone but the other moved and reinserted in 10%. Following one of the T_1 offspring with a single copy of the transposon, the remaining copy was retained in 50% of the T_2 generation but was lost in the other 50%. Overall, the frequency of reinsertion into another location was only a fraction of 'jumping' was far less than 'jumping' and disappearing.

The situation was further complicated by the fact that, on numerous occasions, the anythocyanin regulatory gene was also lost independent of the *Ac* element. This contrasts previous studies in the laboratory where inserted genes were stably incorporated and inherited. Simple Mendelian segregation into the next generation could explain the loss of both the Ac and anthocyanin markers; however, loss of only the anthocyanin marker would not be expected prior to a 'jumping' event.

Visible mutations were rare. Of the hundreds of T_0, T_1, T_2 and T_3 plants grown and observed, there were few abnormalities noted, aside from the variegation described above and shown in Figure 8-4. In many cases, it appeared that apical dominance had been reduced as extra branching occurred on the plants. However, this could not be associated with a 'jumping' event (changes in Southern hybridization banding patterns). On numerous occasions, the first leaves on T_1 and T_2 seedlings were unusually small and leathery. However, after several further nodes of growth, the new leaves appeared normal. No genes were identified by inverse PCR prior to the end of the funding period.

A poster on transposon mutagenesis in peas was presented at the Fifth Canadian Plant Tissue Culture and Genetic Engineering Workshop held at Saskatoon, SK, May 12-15, 2001. A copy is attached.

(f) Innovation Fund Acknowledgment

The following Journal Articles (appended to report) acknowledge funding from AFIF:

1. Polowick, P.L., A. Vandenberg, and J.D. Mahon 2002. Field assessment of outcrossing from transgenic pea (*Pisum sativum* L.) plants. Transgenic Research **11**, 515-519.
2. Després, C., C. DeLong, S. Glaze, E. Liu, and P.R. Fobert. 2000. The Arabidopsis NPR1/NIM1 protein interacts with a subgroup of the TGA family of bZIP transcription factors. The Plant Cell **12**, 279-290.

The following review article (appended to report) acknowledges funding from AFIF.

1. Fobert, P.R. Systemic Acquired Resistance. PBI Bulletin, Jan 2001. pp3-7.

The following Oral Presentations, Posters and Conference Proceedings acknowledge funding from AFIF (copies of poster presentations are appended to report).

1. Fobert, P. Control of Pathogen-Induced Gene Expression in Plants: How to regulate the regulator. Department of Biology, University of Regina, February 8, 2002.
2. Després, C. and P. Fobert. NPR1/NIM1 enhances the DNA binding activity of TGA7, a novel member of the Arabidopsis TGA/OBF transcription factors. The International Society of Molecular Plant-Microbe Interaction Meeting, Madison, WI July 10-14, 2001. Poster.
3. Polowick, P. Pollen flow – Legumes (Peas). Fifth Canadian Plant Tissue Culture and Genetic Engineering Workshop. Saskatoon, SK, May 12-15, 2001.
4. Polowick, P, Anderson, M., Baliski, D. and Mahon, J. Transposon mutagenesis for genetic improvement of pulse crops. Fifth Canadian Plant Tissue Culture and Genetic Engineering Workshop. Saskatoon, SK, May 12-15, 2001. Poster.
5. Mahon J.D., P.R. Fobert, P.L. Polowick, and C. Després. Biotechnology for Saskatchewan Pulse Crop Improvement, Canada-Saskatchewan Agri-Food Innovation Fund Conference, Regina, Nov. 6-8, 2000. Poster.
6. Mahon J.D., P.R. Fobert, P.I. Polowick, Xinjie Xia, Fang Ming Lai. Experiments on Transgene Performance in Pulse Legumes, Biotechnology. Berlin. September 8-11, 2000. Poster.
7. Després, C. Possible Strategies for Engineering Disease Resistance through Manipulation of the NPR1 Signal Transduction Pathway. Workshop on "Towards Engineering Signal Transduction Mechanisms". International Society of Plant Molecular Biology Conference, Québec, June 19, 2000
8. Després, C., C. DeLong, S. Glaze, J. Kalanack and P. Fobert. The Arabidopsis Disease Resistance Protein, NPR1/NIM1, Interacts with a Subgroup of the TGA Family of bZIP Transcription Factors. International Society of Plant Molecular Biology, Quebec, June 18-24, 2000.
9. Fobert, P., C. Després, C. DeLong, S. Glaze and J. Kalanack. The Arabidopsis Disease Resistance Protein, NPR1/NIM1, Interacts with a Subgroup of the TGA Family of bZIP Transcription Factors. Signaling during Biotic and Abiotic Stress in Plants, Keystone Symposium, Sagebrush, NM, February 22-28, 2000. Poster.

10. Mahon, J., Polowick, P., Fobert, P. and Vandenberg, B. New developments in pea biotechnology. Biennial BIC/DNBC & NPIA (National Pea Improvement Association) Meeting. Calgary, Alberta, November 6-13, 1999.
11. Polowick, P.L. and Mahon, J.D. Development of herbicide-tolerant transgenic peas—From the lab to the field. Plant Biology Canada '99, Meeting of the Canadian Society of Plant Physiologists, Saskatoon, SK, June 19-23, 1999.
12. Mahon, J.D. and Polowick, P.L. Analysis of herbicide-tolerance in transgenic peas: From the field to the lab. Plant Biology Canada '99, Meeting of the Canadian Society of Plant Physiologists, Saskatoon, SK, June 19-23, 1999.
13. Fobert, P. Assessing the Potential of Genetic Engineering Induced Resistance in Pulses. Pulse Crop Research Workshop, Delta Bessborough, November 28, 1998.
14. Polowick, P.L. and Mahon, J.D. Development of herbicide-tolerant transgenic peas: from the lab to the field. Second Pulse Crops Research Workshop, Saskatoon, November 27-28, 1998.
15. Mahon, J.D. and Polowick, P.L. Development of herbicide-tolerant transgenic peas: from the field to the lab. Second Pulse Crops Research Workshop, Saskatoon, November 27-28, 1998.
16. Polowick, P.L. and Mahon, J.D. *Agrobacterium*-mediated genetic transformation of pea (*Pisum sativum*). Annual Meeting of the Canadian Botanical Association, Saskatoon, June, 1998.